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Identification of Four Additional Myoinhibitory Peptides (MIPs) From the Ventral Nerve Cord of *Manduca sexta*

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Four new myoinhibitory peptides were isolated and identified from the ventral nerve cord of adult *Manduca sexta*. The new peptides are related to two previously identified myoinhibitory peptides also isolated from adult *M. sexta*, Mas-MIP I and Mas-MIP II. The sequences of the new peptides are APEKWAAFHGSWamide (Mas-MIP III), GWNDMSSAWamide (Mas-MIP IV), GWQDMSSAWamide (Mas-MIP V), and AWSALHGAWamide (Mas-MIP VI). Mas-MIPs III-VI were found to inhibit spontaneous peristalsis of the adult *M. sexta* anterior hindgut (ileum) in vitro. Arch. Insect Biochem. Physiol. 48:121–128, 2001. **Published 2001 Wiley-Liss, Inc.**†

Key words: *Manduca sexta*; myoinhibitory peptide; myoinhibiting peptide; allatostatin; prothoracicostatin

INTRODUCTION

Members of a diverse range of neuropeptide families can modulate the contractile activity of insect visceral muscle. Schoofs et al. (1991) reported the identification of a novel myoinhibitory peptide from *Locusta migratoria* with the primary structure AWQDLNAGWamide. This peptide, termed Locustamyoinhibiting peptide (Lom-MIP), was found to suppress the spontaneous contractile activity of the hindgut and oviduct of the locust. Subsequently, we reported the identification of two similar myoinhibitory peptides (Mas-MIP I and II) from the ventral nerve cord of adult *Manduca sexta* (Blackburn et al., 1995a). Mas-MIPs I and II, with primary structures AWQDLNSAWamide and GWQDLNSAWamide, respectively, were found to inhibit spontaneous contractions of the anterior hindgut of the adult moth in vitro. Soon after Mas-MIP I and II were reported, four nonapeptides with obvious homology to the previously described MIPs were described from the cricket, *Gryllus bimaculatus* (Lorenz et al., 1995a). However, these peptides were isolated based on their ability to inhibit juvenile hormone synthesis in the cricket, and were classified as allatostatins (Grb-AST B1-B4). In addition to the

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Abbreviations used: ESMS, electrospray ionization mass spectrometry; MeCN, acetonitrile; TFA, trifluoroacetic acid.

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allatostatic activity of "MIPs" in *G. bimaculatus*, Hua et al. (1999) have reported that a peptide identical to Mas-MIP I has been isolated from *Bombyx mori* based on its prothoracicostatic activity; this peptide has accordingly been named Bom-PTSP.

Here we report the identity of four additional members of the "MIP" family of neuropeptides identified from extracts of adult *M. sexta* ventral nerve cord. Like Mas-MIPs I and II, Mas-MIPs III-VI were found to inhibit spontaneous contractile activity of the *M. sexta* anterior hindgut in vitro.

MATERIALS AND METHODS

Extraction

Approximately 350 abdominal ventral nerve cords of adult *M. sexta* were dissected and immediately frozen in 10 ml of extraction solvent (methanol/water/acetic acid; 90:9:1) on dry ice. The nerve cords were then homogenized in the extraction solvent at room temperature and clarified by centrifugation at 10,000*g* for 3 min. The pellet was re-extracted with 5 ml of additional extraction solution, centrifuged, and the supernatant combined with that of the first extraction.

HPLC

The extract was diluted 10-fold with 0.1% aqueous trifluoroacetic acid (TFA) and loaded onto a C18 solid-phase extraction cartridge. The cartridge was washed with 3 ml of 0.1% TFA, and eluted with 3 ml of 40% acetonitrile (MeCN) in 0.1% aqueous TFA. The myoinhibitory peptides were purified from this eluate by reverse-phase HPLC using a four-step procedure as follows.

Method 1: Vydac C4 column (4.6 × 250 mm, 5 μm packing) eluted with a gradient of isopropanol in 0.1% TFA (0-10% isopropanol in 10 min, 10–60% isopropanol over the next 60 min).

Method 2: Brownlee C8 column (2.1 × 250 mm, RP-300 packing) eluted with a gradient of MeCN in 50 mM sodium phosphate buffer (pH 6.0; 0–15% MeCN in 5 min, 15–50% MeCN over the next 42 min).

Method 3: Brownlee C8 column (2.1 × 250 mm, RP-300 packing) eluted with a gradient of MeCN in 50 mM H_3PO_4 (0–15% MeCN in 5 min, 15–50% MeCN over the next 42 min).

Method 4: Vydac C4 column (4.6 × 250 mm, 5 μm packing) eluted with a gradient of MeCN in 0.1% TFA (0–10% MeCN in 10 min, 10–40% MeCN over the next 60 min).

Separations were performed at 25°C on a Hewlett-Packard Model 1090 HPLC equipped with a diode array detector. The nerve cord extract and subsequent HPLC fractions were diluted with sufficient aqueous mobile phase to lower the organic solvent content below 10% prior to loading onto the reverse-phase columns. The diluted samples were then loaded via an outboard 9-ml sample loop and injection valve.

As discussed in our earlier report, the isolation of the MIPs was a serendipitous by-product of our search for kinins in the ventral nerve cord of *M. sexta*. We utilized a strategy involving screening our initial chromatographic fractions by an in vitro Malpighian tubule assay (Blackburn and Ma, 1994) followed, in subsequent HPLC fractionations, by selectively purifying peptides whose UV spectra indicated the presence of tryptophan. The second derivative of such spectra will show minima at 280 and 290 nm, with a maximum at 285 nm (Jaffe et al., 1986; Blackburn et al., 1995a,b). The Mas-MIPs were isolated because the MIPs have similar chromatographic behavior to kinins and were co-mingled with diuretic activity following our initial fractionation, and because both kinins and MIPs contain tryptophan.

Characterization of Purified Peptides

Purified peptides were sequenced by automated Edman degradation on an Applied Biosystems 477A pulsed-liquid sequencer. Molecular weights of the peptides were determined by capillary HPLC-electrospray ionization mass spectrometry (ESMS) on a Finnigan-Mat TSQ 700.

Preparation of Synthetic Peptides

Synthetic peptides were prepared as C-terminal amides on a Milligen/Biosearch 9600 synthesizer using *t*-Boc protocols supplied by the manufacturer. Standard protective groups were used. Product peptides were cleaved from the resin and deprotected with HF/anisole/*p*-cresol/*p*-thiocresol, washed with methyl-*t*-butyl ether, and lyophilized from water. The synthetic peptides were purified on a 1 × 30 cm Dynamax-300A C8 column and eluted with a linear gradient (0.8% per min) of MeCN in 0.1% TFA.

Bioassay for Myoinhibitory Activity

In adult *M. sexta*, the anterior portion of the hindgut, which we will refer to as the ileum, is a long and convoluted intestine that joins the midgut at its anterior end, and opens into the sac-like rectum at its posterior end. The ileum was chosen because of its spontaneous contractile activity, which is generally quite stable for long periods, and because Lom-MIP was shown to be active on the hindgut of *Locusta* (Schoofs et al., 1991). Synthetic peptides were assayed for myoinhibitory activity on freshly dissected ilea from 2–5-day-old adult male *M. sexta*. One end of a 1-cm segment of the posterior ileum (nearest the rectum) was pinned into a Sylgard-lined incubation dish, while the free end was attached, by means of a fine nylon monofilament, to a home-made isotonic transducer. The tension applied by the transducer to the preparation was approximately 10 mg (determined by attaching the lever of the transducer to the pan of an analytical balance). The ileum was washed continuously with oxygenated *Manduca* saline (Kataoka et al., 1989) containing 0.5% glucose. Saline was gravity fed into the incubation dish while the saline level was maintained by drainage via a vacuum line. Peptides in *Manduca* saline were also gravity fed into the dish through a separate line, while the normal wash was interrupted. Each of the Mas-MIPs, including Mas-MIPs I and II, were assayed at 0.1-, 1.0-, and 10-nM concentrations. After an initial 15–20 min period of equilibration, each ileum was exposed to the three concentrations of peptide sequentially (in ascending order of concentration); the length of exposure to each concentration was 4 min. Preparations were used once, for a single assay of a single peptide. Each peptide was assayed 6–8 times.

RESULTS

Fractionation of the nerve cord extract by HPLC Method 1 is illustrated by the chromatogram in Figure 1A. Fractions eluting between 29 and 39 min caused the greatest increase in secretion by Malpighian tubules in vitro (recall that our initial intent was to isolate and identify kinins). Within this range of fractions, the region between 31 and 35 min contained a number of peaks with UV spectra which indicated the presence of tryptophan. Two myoinhibitory peptides, Mas-MIP I and Mas-MIP II, identified from fractions 33–35, were described in our earlier report (Blackburn et al., 1995a). Shown in Figures 1B and C are chromatograms of HPLC Method 2 separations of fractions 31–32 and 33, respectively. Four tryptophan-containing peptides were detected by their distinct UV spectra and further purified by HPLC Methods 3 and 4. Edman degradation of these peptides revealed that all were related to the previously described Mas-MIPs I and II. We, therefore, designated the newly identified peptides Mas-MIP III, IV, V, and VI. The sequences of all the Mas-MIPs identified thus far are shown in Figure 2. The sequences of the four new MIPs were consistent with molecular mass determinations by ESMS, and amidated synthetic peptides based on these sequences were found to co-elute with their native counterparts by HPLC Method 4 (data not shown).

The effects of synthetic Mas-MIP III, IV, V, and VI on isolated posterior ilea of adult *M. sexta* are shown in Figure 3. At a 10-nM concentration, all of the newly identified Mas-MIPs rapidly inhibited or abolished spontaneous contractions of the posterior ileum. Contractions quickly resumed when preparations were washed with saline. Results of many such assays are illustrated in Figure 4. Based on these results, Mas-MIP I is the most potent of the Mas-MIPs in inhibiting peristalsis of the ileum in vitro, consistently inhibiting contractions at a concentration of 1 nM. The remaining MIPs appear to require higher concentrations to consistently inhibit peristalsis; in particular, Mas-MIPs III and VI would seem to be at least 10-fold less potent than Mas-MIP I. Considerably more effort would be needed to accurately determine the relative potencies of the six peptides.

DISCUSSION

Here we report the identities of four new members of the MIP family of neuropeptides. A total of six MIPs have now been described from the ventral nerve cord of adult *M. sexta*. With the exception of the N-terminally extended dodecapeptide Mas-MIP III, all members of this family are amidated nonapeptides. The most striking common feature of the MIPs are tryptophan resi-

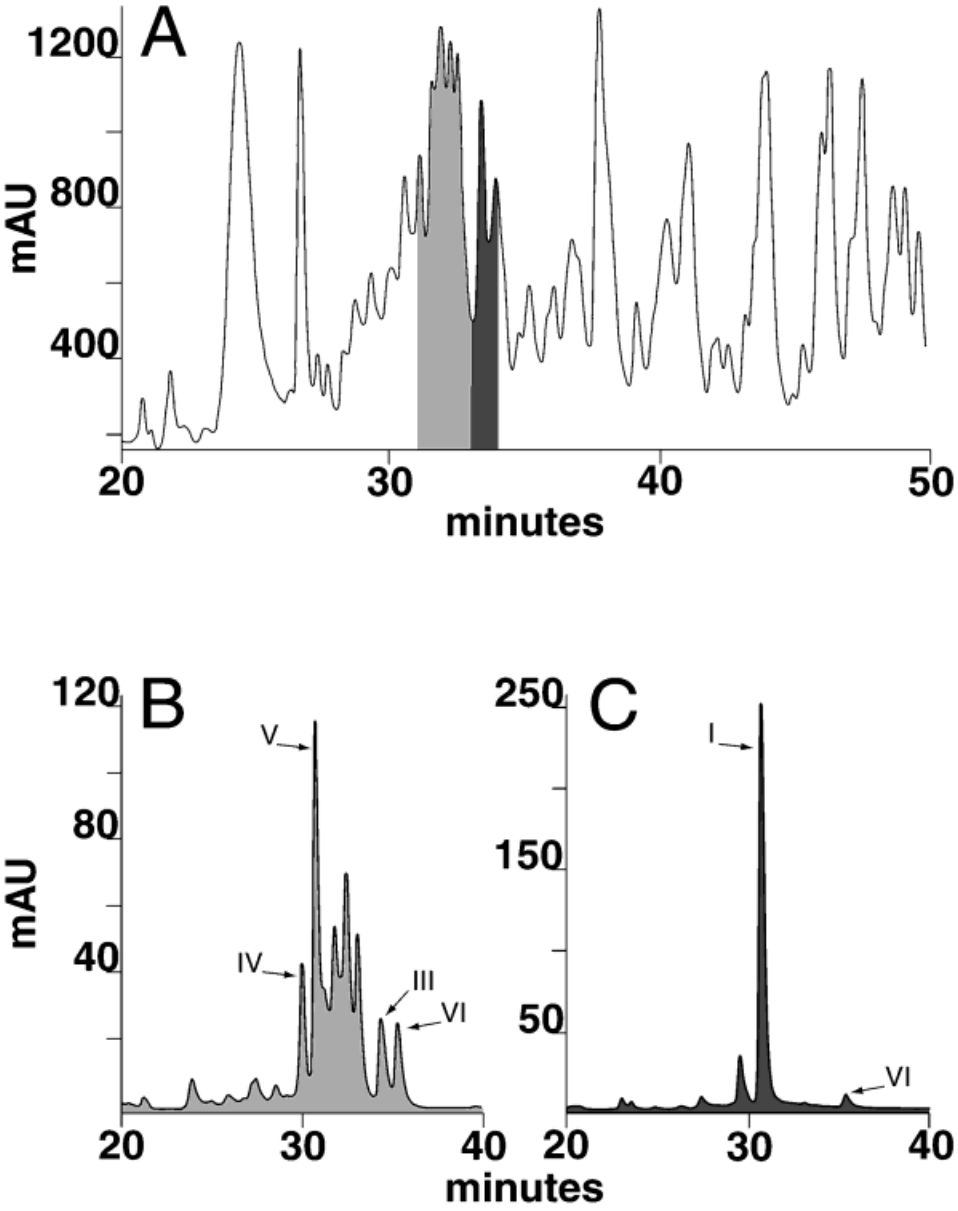

Fig. 1. Purification of Mas-MIPs III-VI. **A:** Chromatogram of the crude extract of ca. 350 ventral nerve cords fractionated by HPLC Method 1. Fractions 31–32 (light shading) and fraction 33 (dark shading) were then re-fractionated by HPLC Method 2. **B:** HPLC Method 2 fractionation of fractions 31–32 from HPLC Method 1. Peaks corresponding to MIPs III–VI are labeled. **C:** HPLC Method 2 fractionation of fraction 33 from HPLC Method 1. This fraction contained the previously identified Mas-MIP I, as well as a portion of Mas-MIP VI, which was split between fractions 32 and 33 in Method 1. All the peaks in B and C whose UV spectra indicated the presence of tryptophan were further purified to homogeneity by HPLC Methods 3 and 4. Absorbance is at 210 nm for A, and 280 nm for B and C.

dues at positions 2 and 9 (again, with the exception of Mas-MIP III). Based on UV absorbance at 280 nm measured during the third chromatographic step (Method 3), Mas-MIP I is the most abundant of the Mas-MIPs. The quantity of Mas-MIP V appears to be approximately 50% that of Mas-MIP I, while the remaining Mas-MIPs all appear to be present at levels approximately 20% that of Mas-MIP I.

All the Mas-MIPs inhibit peristalsis of the posterior ileum of adult *M. sexta* at concentrations equal to or below 10^{-8} M. Although the dose-response data presented in this study are limited, it seems clear that Mas-MIP I is the most potent,

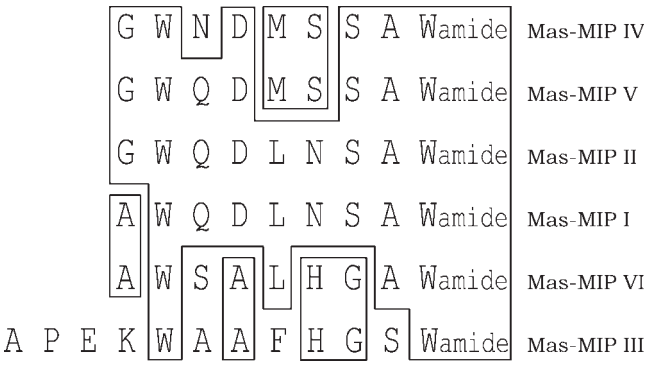

Fig. 2. The aligned sequences of the Mas-MIPs.

while Mas-MIPs III and VI are the least potent inhibitors of ileal peristalsis. Aside from the N-terminal extension of Mas-MIP III, the sequences of Mas-MIPs III and VI share features that deviate markedly from the other Mas-MIPs (see Fig. 2). The molluscan peptides APGWamide and GWamide from *Lymnea stagnalis* (Smit et al., 1992) and *Sepia officinalis* (Henry et al., 1997), respectively, have been shown to be myoinhibitory, suggesting that the C-terminus of the MIPs may be more critical than the N-terminus for myoinhibitory activity. The fact that the N-terminally extended Mas-MIP III retains activity may also indicate that the N-terminus is less critical for activity.

The distribution of Lom-MIP in *L. migratoria* has been studied immunocytochemically (Schoofs et al., 1996). Lom-MIP-like immunoreactivity (Lom-MIP-LI) was detected in neurosecretory cells of the brain, subesophageal ganglion, metathoracic ganglion, and all abdominal ganglia except the terminal ganglion. Lom-MIP-LI was detected in nerves innervating the oviduct, heart, hindgut,

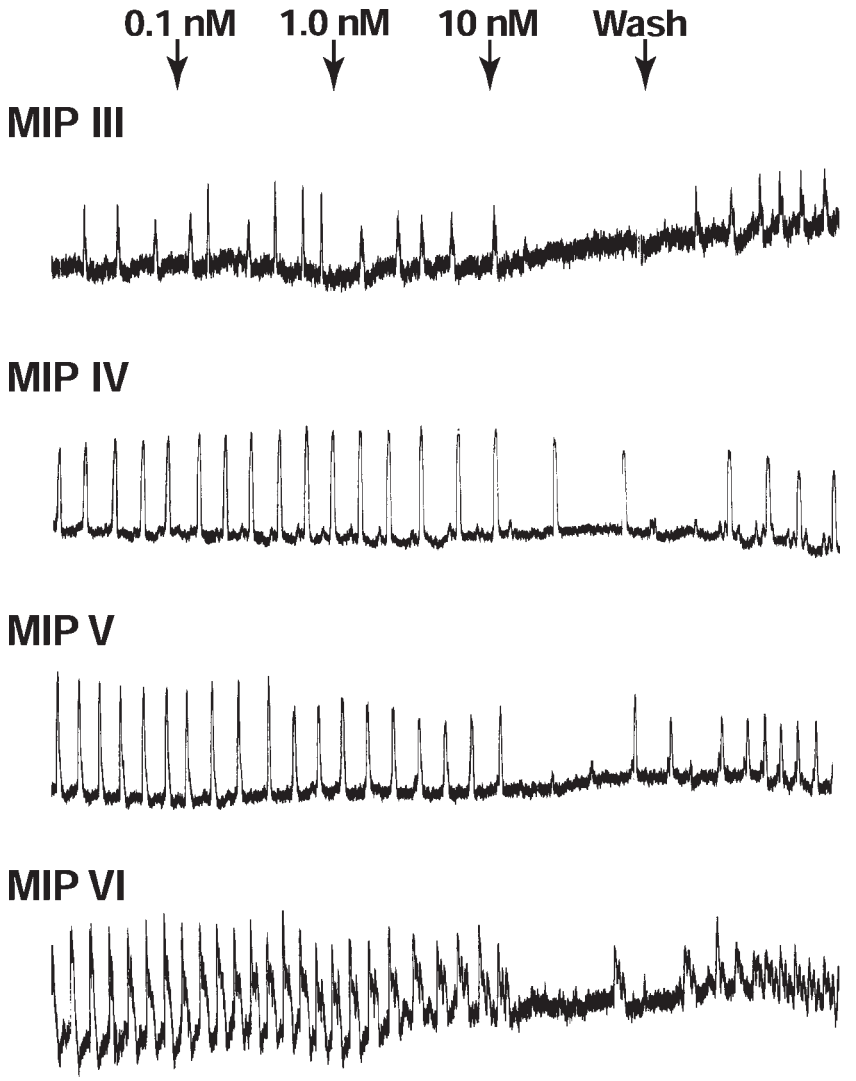

Fig. 3. Representative responses of isolated posterior ilea from adult *M. sexta* to Mas MIPs III–VI. Concentrations of 0.1, 1, and 10 nM were tested sequentially, followed by a saline wash. All four MIPs exhibit inhibitory activity at 10^{-8} M concentrations. The inhibitory activity of all four peptides was reversible.

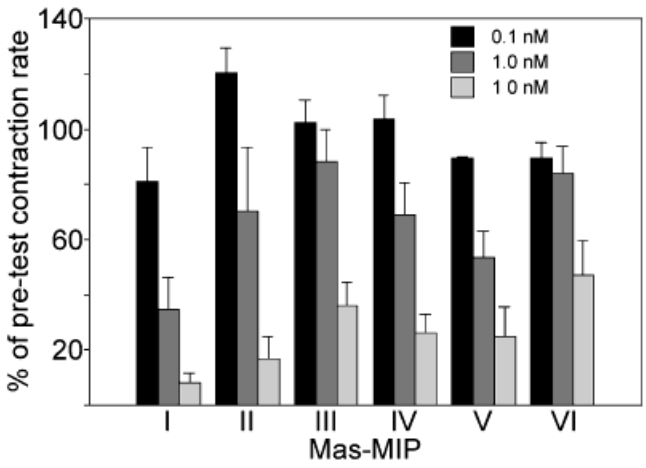

Fig. 4. The effects of Mas-MIPs I-VI on the contraction rate of the posterior ileum in vitro. The rate of contraction for each concentration of peptide was expressed as a percentage of the rate prior to the application of the lowest concentration of peptide. Data for each peptide represent the mean (± SEM) of 6–8 assays.

and both the glandular corpus cardiacum and corpus allatum.

Reports of "MIPs" from *G. bimaculatus* and *B. mori* indicate that these peptides may regulate not only the activity of visceral muscle, but may also play a key role in the regulation of juvenile hormone and ecdysteroid synthesis. The four peptides from *G. bimaculatus* were isolated based on their ability to inhibit juvenile hormone synthesis in the cricket, and have accordingly been classified as allatostatins; Grb-AST B1-4 (Lorenz et al., 1995a). Subsequently it was shown than Grb-AST B1 inhibited ovarian ecdysteroidogenesis (Lorenz et al., 1997) and lowered ecdysteroid titers in an in vivo study (Lorenz et al, 1998). The peptide from *B. mori*, identical to Mas-MIP I, was isolated based on its prothoracicostatic activity, and named Bom-PTSP (Hua et al., 1999). The prothoracicostatic activity of Bom-PTSP/Mas-MIP I in a lepidopteran insect suggests that similar activity might be encountered in *M. sexta*. In addition to the activities described above, it has been shown recently in *L. migratoria* that Lom-MIP inhibits the release of adipokinetic hormone evoked by the phosphodiesterase inhibitor IBMX (Vullings et al., 1999).

It is interesting to note that although structurally unrelated to the MIP family of peptides, the "cockroach allatostatins" have also been shown to possess both myoinhibitory and allatostatic activity. These peptides, which share a C-terminal sequence of YXFGLamide, appear to occur ubiquitously among insects (Pratt et al., 1989, 1991; Woodhead et al., 1989, 1994; Duve et al., 1993, 1997a; Belles et al., 1994; Veelaert et al., 1996; Lorenz et al., 1995b; Davis et al., 1997; Veenstra et al., 1997). The YXFGLamides appear to have allatostatic activity that is confined to cockroaches (Pratt et al., 1989, 1991; Woodhead et al., 1989, 1994; Belles et al., 1994) and the cricket, *Gryllus bimaculatus* (Lorenz et al., 1995b). By comparison, the myoinhibitory effects of these peptides on visceral muscles have been documented on a diverse range of insects (Lange et al., 1993; Duve and Thorpe, 1994; Duve et al., 1997b; Veelaert et al., 1996; Rankin et al., 1998). Although the allatostatic activity of "MIP" peptides has not been tested beyond *G. bimaculatus*, the correlation of myoinhibitory and allatostatic activity in these disparate peptide families deserves further investigation.

A Blast search (Altshul et al., 1997) of the databases with Mas-MIP sequences revealed a probable prepro-MIP peptide from *Drosophila melanogaster* of 211 amino acids containing 5 MIP-like sequences (GenBank accession number AAF49354; Adams et al., 2000). Each of the possible Drm-MIP peptides is flanked by the paired basic amino acid residues necessary for proteolytic cleavage, and the presence of glycine following the C-terminal tryptophan residue of each suggests the mature peptides are amidated. Further searches using the putative *D. melanogaster* prepro-MIP as a template revealed an apparent relationship (41% sequence identity over 70 residues) to prepro-APGWamide. Prepro-APGWamide a 219 amino acid precursor of the aforementioned myoinhibitory peptide of the snail, *L. stagnalis* (Smit et al., 1992), contains ten copies of the APGWG prohormone, which are further processed to APGWamide. Seven copies are contained in a region of contiguous repeats of the sequence KRAPGWG, resulting in a spacing of tryptophan residues identical to the MIPs. Thus, it is plausible that the insect "MIP" peptides and APGWamide of the snail share a common ancestral origin. In our earlier report, we noted that Mas-MIPs I and II resembled the N-terminus of the vertebrate galanins. Sequences of the newly identified MIPs diverge significantly from the gal-

anins, as do the sequences of Lom-MIP and the Grb-ASTs. Moreover, the *D. melanogaster* prepro hormone does not bear any resemblance to the galanins or their precursors. It appears that sequence similarities between Mas-MIP I and II and the galanins are probably coincidental.

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